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Effect of biochar produced from different biomass sources and at different process temperatures on methane production and ammonia concentrations *in vitro*

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ABSTRACT

The effects of different biochars on *in vitro* rumen gas production and fermentation characteristics were investigated using a two (biochar inclusion level, 10 and 100 g biochar /kg substrate) x two (process temperature, 550 or 700 ºC) x five (biomass source, *Miscanthus* straw, oil seed rape straw, rice husk, soft wood pellets or wheat straw) factorial design. The amount of biochar included in incubations had no effect on *in vitro* fermentation. Overall, inclusion of biochar reduced total gas production to 0.96 ($P<0.001$) and methane (CH_4) production to 0.95 ($P<0.001$) of that in control (no added biochar) incubations. There were no differences in gas or CH_4 production between the biomass sources used to produce biochar but total gas ($P=0.058$) and CH_4 ($P=0.010$) production were slightly greater when biochar was produced at 700 rather 550 ^{0}C. Addition of biochar to incubations did not change total amounts of volatile fatty acids (VFA) or acetic acid produced during *in vitro* fermentation; however, the amounts of propionate (0.94; $P<0.001$) and butyrate (0.96; $P=0.021$) were reduced when biochar was added to incubations. Process temperature had no effect on VFA produced; however, total VFA and the amounts of acetic and butyric acids produced were influenced by biochar biomass source. Ammonia concentrations at the end of incubations were overall 0.84 of control concentrations ($P<0.001$)when biochar was added. Both process temperature and biochar biomass source influenced ammonia concentrations which were greater for biochar produced at 700 than 550 ^{0}C; concentrations were lowest for biochar produced from *Miscanthus* straw and greatest for rice husk with oil seed rape straw, soft wood pellets and wheat straw intermediate. Adding biochars with a range of compositions to *in vitro* assays produced only small reductions in CH_4 production. However, the absence of any negative effects of biochar coupled with the observed reduction in ammonia concentrations makes it possible that including biochar in livestock feed could be a practical means of applying biochar to pasture and soil.

Keywords: biochar; process temperature; biomass source; *in vitro* methane production; ammonia concentration

1. Introduction

Methane (CH_4) emissions arising from the enteric fermentation of feed by ruminant livestock is an important contributor to global greenhouse gas emissions. For example, in 2014 in the United Kingdom (Department of Energy and Climate Change, 2016), enteric CH_4 emissions were estimated to account for 23.8 Mt carbon dioxide equivalents or 48% of total greenhouse gas emissions from the agriculture sector. There is therefore a need to develop strategies to mitigate CH_4 emissions and Hristov et al. (2013) distinguished those that directly address enteric fermentation; that focus on manure management and those that target animal husbandry (where animal husbandry includes genetics, health and fertility).

Manufacture and use of biochar has the potential to mitigate the impact of agriculture on greenhouse gas emissions in various ways. Biochar is the solid, carbon-rich product of biomass pyrolysis; the controlled heating of biomass at high temperature with deliberate exclusion of oxygen. Converting degradable biomass into recalcitrant biochar before adding it to soil demonstrably sequesters carbon into the land. It has also been found to suppress soil-based emissions of CH_4 and nitrous oxide (Sohi et al., 2010; Gurwick et al., 2013). The reason for the latter effects remain uncertain, but probably involve the general porosity of biochar and the large negative charge gradually developed across its large surface area (Kammann et al. 2017).

The inclusion of biochar in ruminant diets has been investigated for two reasons. First, biochar may reduce enteric CH_4 emissions (Leng et al., 2012; Hansen et al. 2012; Calvelo Pereira et al. 2014) and secondly, faecal excretion of dietary biochar may provide an

effective means of transferring biochar into slurry or on to pasture (Calvelo Pereira et al. 2014; Joseph et al. 2015). Responses to the inclusion of biochar in rumen *in vitro* assays have been variable ranging from no effect (Calvelo Pereira et al. 2014) to a 13% reduction (Leng et al. 2012). As the properties of biochar are dependant on both the temperature of pyrolysis and the biomass source from which it was prepared, such variation is not surprising. The objective of the current work was therefore to determine whether biochar suppressed CH_4 production *in vitro* and by using a range of biochars with defined chemical and physical compositions to investigate the attributes of biochar responsible for suppressing CH_4 *in vitro*.

2. Material and methods

This experiment was conducted at Scotland's Rural College (SRUC) Beef and Sheep Research Centre in Edinburgh in 2013. The experimental protocol was approved by SRUC's Animal Welfare and Ethical Review Body, the Animal Experiments Committee and was conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act, 1986.

2.1. Biochar

The experiment used 10 standard biochars provided by the UK Biochar Research Centre, University of Edinburgh. These were manufactured in a scalable 20 minute process optimised for research use, comprising a rotating kiln heated indirectly and electrically. The biochars differ by their source biomass (five granular biomass sources: rice husks and pelleted *Miscanthus* straw, oil seed rape straw, wheat straw or soft wood) and peak processing temperature (550 or 700 ºC). A summary of biochar composition is given in Table 1. Full details of biochar production and composition can be found at (http://www.biochar.ac.uk/standard_materials.php; accessed 07/02/2017). To ensure that

particle size was small enough for inclusion in assays and to avoid gross differences between biochars, the material used in the assay was the fraction that passed through a 2 mm screen.

2.2. Experimental design

A 2 (biochar inclusion) x 2 (process temperature) x 5 (biomass source) factorial design was used where the factors were: biochar addition (10 or 100 g biochar/kg substrate fresh weight); biochar process temperature (550 or 700 ºC) and biomass source (*Miscanthus* straw, oil seed rape straw, rice husk, soft wood pellets or wheat straw). Each of the 20 individual treatments was incubated in triplicate in each replicate (week of experiment). Within each replicate, control samples which contained substrate but no added biochar and blank samples without substrate or biochar were also included in triplicate giving a total of 66 incubations on each replicate. There were a total of four replicates of the above carried out at 14 day intervals.

2.3. Rumen fluid inocula

To provide biological variation in rumen inocula, rumen samples were obtained from a group of cross-bred beef cattle (approximately 16 months in age) fed *ad libitum* a diet consisting of 500 g forage and 500 g concentrate /kg dry matter (DM). Steers were fed once daily and rumen samples were obtained at approximately 08.00 h before fresh feed was offered. Rumen samples were obtained using a stomach tube (16 × 2700 mm) introduced into the oesophagus via a nostril and then passed down to the rumen. Samples were immediately strained through two layers of muslin and transported in insulated flasks under anaerobic conditions to the laboratory and used as inocula within one hour of collection. For each replicate of the experiment, three different rumen inocula were prepared. Where possible

each inoculum (total volume 300 mL) was derived from an individual animal. On two occasions, sample volume from an individual animal was inadequate and therefore, a composite sample was produced by mixing samples from two animals. No animal contributed rumen fluid on more than one occasion. Each of the triplicate incubations noted above therefore contained three different rumen fluid inocula: that is of the 66 incubations per replicate, 22 each contained rumen fluid from a different rumen fluid inoculum. Thus 12 different inocula were used in total for the four replicates.

2.4. In vitro gas production

Incubations took place in 160 mL serum bottles which contained 400 mg feed substrate (343 mg DM) and biochar (4 or 40 mg) as appropriate. The feed substrate consisted of a mixture (g/kg fresh weight) of hay (500), barley (400) and rapeseed meal (100) ground to pass through a 2 mm screen. The feed substrate was chosen to have a similar forage to concentrate ratio and nutrient profile to the diet of the cattle used as rumen fluid donors and thus was also typical of diets fed to finishing beef cattle. Feed substrate was analysed for DM, crude protein, acid hydrolysed ether extract and neutral cellulose and gamanase digestibility according to Ministry of Agriculture Fisheries and Food (1992). Chemical composition was: DM, 857 g/kg and (g/kg DM); crude protein, 105; acid hydrolysed ether extract, 19; neutral cellulase plus gamanase digestibility, 760 and estimated metabolisable energy, 11.2 MJ/kg DM.

The rumen fluid was mixed with buffer-mineral solution, prepared as described by Menke et al. (1979) at a ratio 1:3 (v/v), rumen fluid: buffer. Rumen fluid: buffer mixture (40 mL / bottle) was dispensed under a stream of carbon dioxide, and the bottles were closed with a butyl rubber stopper and placed in a water bath at 39 ºC for 24 h. Contents were thoroughly mixed periodically throughout the 24 h.

120 *2.5. Analytical methods*

121 Cumulative gas production during the 24 h incubation was measured by pressure
122 using a manual pressure transducer (Digitron 2023P, Digitron, Torquay, Devon, UK). The
123 pressure values were converted to the volumes of gas produced using the equation below
124 determined for local laboratory conditions.

125 $V = (P - 11.58) / 7.55$

126 where V = gas volume (mL) and P = pressure (mbar)

127 The gas produced due to fermentation of the feed substrate was corrected for gas
128 produced in appropriate blank incubations. After measurement of pressure, 20 mL gas
129 samples were transferred in duplicate to evacuated head-space vials and CH_4 was analysed by
130 gas chromatography (Agilent 7890, Agilent Technologies**,** Cheshire, UK**)** using a HayeSep Q
131 (80/100), 0.25m x 1mm internal diameter column with helium as carrier gas and detection by
132 flame ionisation using authenticated standards. At the end of the incubation, the bottles were
133 uncapped and pH measured immediately. Samples for volatile fatty acid (VFA) analysis (5
134 mL) were de-proteinized by adding 1 mL metaphosphoric acid (215 g/L) and 0.5 mL
135 methylvaleric acid (10 g/L) as an internal standard. These samples were stored at -20 °C
136 between collection and analysis. VFA concentrations were determined by HPLC as described
137 in Rooke et al. (1990). Samples for analysis for ammonia (NH3-N) were diluted 1:1 (v/v)
138 with 1M-HCl and analysed using the phenol-hypochlorite method of Weatherburn (1967)
139 adapted for 96 well plates with absorbance measured at 625 nm.

140 *2.6. Calculations and statistical analyses*

141 Amounts of total gas, CH_4 and VFA produced were corrected for amounts produced
142 in blank incubations and expressed either as the total amount produced or per g substrate DM

incubated. To assess the overall effect of biochar inclusion, values were expressed as a proportion of the control value for each of the 12 rumen fluid inocula and a single sample t-test used to determine if the overall mean value differed from one (control value). Differences between biochar treatments were analysed according to a factorial design using the Linear Mixed Models procedure of GenStat (version 11.1 for Windows; VSN International Limited). The model included the fixed effects of biochar inclusion, process temperature and biomass source and their interactions. The different replicates and rumen fluid inocula (within replicate) were included as random factors. Where significant differences ($P<0.05$) were detected between biomass sources, differences between means were identified using least significant differences.

3. Results

3.1. Rumen inocula

Using rumen fluid inocula obtained from different animals (Table 2) to inoculate the *in vitro* incubations achieved the objective of producing fermentations differing ($P<0.001$) not only in the extent (amounts of total gas, CH_4 and VFA produced) but also in the type of fermentation (VFA molar proportions and NH3-N concentration).

3.2. Gas and CH_4 production

The amount of biochar included in incubations had no effect on *in vitro* fermentation and there were also no interactions between the amount of biochar included in assays, process temperature used to produce biochar or the source of biochar. Therefore Tables 3 and 4 report only the main effects of biochar biomass source and process temperature. Overall, inclusion of biochar in assays reduced total gas production to 0.96 (SEM 0.003, $P<0.001$) and CH_4

production to 0.95 (SEM. 0.008, P<0.001) of that in control (no added biochar) incubations. There were no differences (Table 3) in gas or CH_4 production between the biomass sources used to produce biochar but total gas (P=0.058) and CH_4 (P=0.010) production were slightly greater when biochar was produced at 700 rather than 550 ^{0}C. When expressed as a ratio of total gas production, CH_4 produced /mL total gas was 0.98 (SEM 0.040, P=0.021) of that in control (no added biochar) incubations and lower for biochar produced at 550 than 700 ^{0}C (P=0.003). However there were no differences between biomass sources.

3.3 VFA production

Overall addition of biochar to incubations did not change total amounts of VFA or acetic acid produced during *in vitro* fermentation; however, the amounts of propionate (0.94; SEM 0.011, P<0.001) and butyrate (0.96; SEM 0.015, P=0.021) were reduced when biochar was added to incubations. Process temperature had no effect on VFA produced during *in vitro* incubations (Table 4). However, total VFA and the amounts of acetic and butyric acids produced were influenced by biochar biomass source with extent of production ranked; lowest for *Miscanthus* straw and highest for rice husks with oilseed rape straw; wheat straw; soft wood pellets intermediate.

3.4. NH3-N and pH

NH3-N concentrations at the end of incubation were overall reduced to 0.84 of control concentrations (SEM 0.022, P<0.001) by addition of biochar. Both process temperature and biochar biomass source influenced NH3-N concentrations (Table 4). Concentrations were greater for biochar produced at 700 than 550 ^{0}C. NH3-N concentrations for biomass sources were lowest for *Miscanthus* straw and greatest for rice husk with oil seed rape straw, soft wood pellets and wheat straw intermediate (in order of ascending concentration). Although

(Table 4) there were significant differences in final incubation pH between treatments, these differences were small with mean values ranging from 6.51 to 6.55.

4. Discussion

Including biochar in *in vitro* rumen fluid incubations reduced total gas, VFA and CH_4 production to a limited extent and NH3-N concentrations to a greater extent.

4.1 Source of rumen fluid

When using the *in vitro* gas production technique, it is usually recommended that the most consistent results are obtained by taking rumen samples after an overnight fast and by combining rumen fluid from a minimum of three animals (Williams 1990). However, such an approach precludes assessment of variation between animals in response and in the current experiment, animal to animal variation was specifically addressed by using 12 different sources of rumen fluid (in most cases from individual animals) in the incubations. Despite the fact that there was substantial animal to animal variation, estimated to be four (gas produced / g substrate DM) to ten (CH_4 produced / g substrate DM) times greater than the variation associated with the biochar treatments imposed, the overall effects of and differences between biochar types were successfully captured. It should be noted that at least some of the animal to animal variation will be related to when feed was last consumed and nutritional quality of feed, as although fresh feed was last offered 24h before rumen samples were obtained, patterns of feed intake would undoubtedly have differed from animal to animal and the nutritional quality of feed from week to week and thus could have led to differences in the chemical and microbiome composition of different inocula.

4.2 Effects of biochar on fermentation

212 Biochar reduced the overall extent of fermentation (gas production) to 0.96 and CH_4
213 emissions to 0.95 of control values. More importantly the ratio of CH_4 to total gas in samples
214 to which biochar had been added was 0.98 of control values. Therefore biochar caused only a
215 small reduction in CH_4 production. In previous *in vitro* studies, variable results have been
216 obtained. At one extreme, Leng et al. (2012) reported a significant reduction in CH_4
217 production to 0.86 of control with no depression in total gas production while Calvelo Pereira
218 et al. (2014) found no overall effect of biochar addition on either gas or CH_4 production.
219 Hansen et al. (2012) reported non-significant reductions by three different biochars of 0.89 to
220 0.92 (of control) for total gas and 0.84 to 0.86 for CH_4 production. Overall CH_4 production
221 when expressed as proportion of total gas production ranged from 0.86 of control values
222 (Leng et al. 2012) to 1.00 (Calvelo Pereira et al. 2014) with Hansen et al. (2012), 0.91 and the
223 present study (0.98) intermediate. Since the biochars used in the above studies were produced
224 from different biomass sources and at different temperatures and little (pH, surface area, C, N
225 and ash; Calvelo Pereira et al., 2014) or no detail (Hansen et al., 2012, Leng et al., 2012)
226 reported on biochar composition, then the range of results is not surprising and difficult to
227 analyse. For example, Leng et al. (2012) prepared biochar at 900 ^{0}C whereas Calvelo Pereira
228 et al. (2014) reported no differences between biochars prepared at 350 or 550 ^{0}C; in the
229 present study, the reduction in CH_4 was greater with biochar prepared at 550 than 700 ^{0}C.
230 Kammann et al. (2017) have suggested that the high pyrolysis temperature used by Leng et
231 al. (2012) could explain why a reduction in CH_4 production was observed. However, the
232 effects of biochar *in vitro* may not be realised *in vivo* because although batch *in vitro* systems,
233 as used in this experiment, are valuable screening tools, they do not reproduce the complex
234 situation *in vivo;* in particular the ability of the rumen microbiome to adapt to novel materials
235 cannot be reproduced and many novel compounds effective *in vitro* are not effective *in vivo*
236 (Hristov et al., 2013).

237 The current study investigated the effects of biochar biomass source and process
238 temperature on *in vitro* fermentation. Overall, the differences between biochars were small
239 but there were consistent effects of biomass source. Biochar prepared from *Miscanthus*
240 reduced gas, CH_4 and VFA production to the greatest extent and biochar prepared from rice
241 husk and soft wood pellets were least effective. In other studies, Hansen et al. (2012) found
242 that straw-derived biochar numerically reduced CH_4 to a greater extent than wood-derived
243 biochar but Calvelo Pereira et al. (2014) reported no difference between wood and crop
244 residue-derived biochars. In addition, no significant correlations were found between mean
245 values for the 10 different biochars for gas and CH_4 production and the compositional
246 information from Table 1. Thus there was no clear evidence for relationships between biochar
247 composition and *in vitro* activity from this or other studies investigating biochar as a means
248 of reducing CH_4; this is possibly not surprising as the same conclusion has been drawn from
249 meta-analysis of the more widely studied area of biochar as a greenhouse gas mitigation
250 strategy when applied to soils (Gurwick et al. 2013).

251 In the light of the above it is difficult to comment on mechanisms by which biochar
252 could reduce CH_4. In the soil and composting environments, the balance between
253 methanogenic archaea and methanotrophic organisms was altered favourably towards
254 methanotrophism rather than methanogenesis with biochar application (Feng et al., 2012;
255 Sonoki et al 2013) but this is unlikely in the anaerobic rumen environment which precludes
256 growth of the aerobic methanotrophs. Other possibilities suggested have included creation of
257 micro-environments by the large surface area of biochar. The absence of any relationship
258 between biochar surface area and CH_4 production in the current study is probably related to two
259 factors. First, the small differences in CH_4 production between biochars made it difficult to
260 discriminate between surface areas of different biochars. Secondly, gross surface area (m^2/g,

Table 1) may not adequately describe the optimum micro-environment for colonisation of biochar by rumen microbes as this may require description of structure not only at the µm scale but also to account for distribution of charged particles (Leng 2014).

4.3 Effects of biochar on NH3-N concentrations

Unexpectedly NH3-N concentrations after 24 h incubation were reduced when biochar was added to incubations. The reduction was most marked in *Miscanthus*-derived biochar (0.58 of control) and biochar prepared at 550 ^{0}C had a greater effect than preparation at 700 ^{0}C. Since the *in vitro* incubation is a sealed system, there are two possible reasons for this difference. First, the differences in NH3-N concentrations could be due to a reduction in proteolysis and deamination of nitrogenous constituents of the feed substrate, increased incorporation of NH3-N into microbial protein or a combination of these two processes. As the differences between treatments in energy supply for microbial growth (gas or VFA production) were small then a reduction in proteolysis / deamination seems more likely but as no direct measurements of these processes were made, then this is speculative. Only Calvelo Pereira et al (2014) also reported NH3-N concentrations *in vitro* with biochar addition but these authors found no differences between treatments. Secondly, in the soil environment, biochar has in some studies reduced leaching of NH3-N (Ding et al. 2010). This has been attributed to the cation exchange capacity of the negatively charged biochar and indeed in laboratory studies, NH3-N is adsorbed (Gai et al. 2014; Winning 2014). In these laboratory studies, the efficacy of biochars in adsorbing NH3-N was inversely related to the temperature at which biochar was produced (increased pyrolysis temperature reduces cation exchange capacity) and to the influence of biomass source on cation adsorption. In the present experiment, NH3-N concentrations were lower when biochar was produced at 550 rather than 700 ^{0}C was included in the assay, which is consistent with the laboratory studies. Thus, an alternative

explanation for the effect of biochar on NH3-N concentrations is that NH3-N was adsorbed by biochar. In the *in vivo* situation, binding of NH3-N by biochar may be beneficial as any NH3-N bound when NH3-N concentrations are high immediately after feeding would be released when NH3-N concentrations declined and therefore would improve synchrony between the supply of NH3-N and energy (from degraded carbohydrates) for rumen microbial protein synthesis.

4.4 Feeding biochar as a means of reducing greenhouse gas emissions from soil and pasture

Including biochar in animal feed as a means of applying biochar to soil and pasture has been suggested (Calvelo Pereira et al. 2014; Joseph et al. 2015) either directly through grazing and defaecation or more likely through the application of animal waste to land, as is standard practice in livestock farming systems. Incorporating biochar into ensiled grass had no adverse effects on the resulting silage (Calvelo Pereira et al. 2014) and Joseph et al. (2015) reported little change in the recalcitrant carbon structure of biochar as it passed through the gut of cattle. Adding biochar up to 100g /kg feed substrate in the current experiment did not adversely effect rumen fermentation. Although direct mitigating effects on *in vitro* CH_4 production were small this suggests that biochar is inert in terms of digestion and using the animal to apply biochar to pasture is possible. However, one factor limiting the use of biochar is that it is inert and therefore including biochar in feed will dilute the energy content of feed and therefore may reduce energy supply to the animal if it cannot increase feed intake to compensate. On the other hand, the reductions in *in vitro* NH3-N concentration observed with biochar may change the balance of nitrogenous constituents in animal excreta as less NH3-N may be absorbed from the digestive tract and excreted as urea in urine, thus decreasing the soluble nitrogenous constituents of manure or slurry. If NH3-N is excreted bound to biochar

it may then contribute to improved N retention in soils as well as and lower emission of ammonia to air.

5. **Conclusions**

Adding biochars with a range of compositions to *in vitro* rumen assays produced small reductions in CH_4 production which may not be reproduced *in vivo*. However, the absence of any negative effects of biochar coupled with the observed reduction in NH3-N concentrations makes it possible that feeding biochar to livestock could be a means of applying biochar to pasture and soil.

Conflict of interest

There is no conflict of interest.

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384

385 **Table 1**

386 Composition of biochars manufactured at different peak temperatures and prepared from different biomass sources (g/kg DM unless stated otherwise)

Source	*Miscanthus* straw pellets		Oil seed rape straw pellets		Rice husks		Soft wood pellets		Wheat straw pellets	
Temperature (^{0}C)	550	700	550	700	550	700	550	700	550	700
Dry matter (g/kg)	982	988	974	964	985	985	985	990	986	978
Ash	122	116	195	219	479	473	125	189	213	238
pH	9.77	9.72	9.78	10.40	9.71	9.81	7.91	8.44	9.94	10.00
C	754	792	689	677	487	473	855	902	683	690
H	24	13	18	11	12	6	28	18	21	12
O	92	70	89	78	25	21	104	60	69	53
N	8	10	16	13	10	9	<1	<1	14	13
P	2	8	3	3	1	5	<1	<1	1	3
K	10	26	29	30	4	6	2	3	16	15
Surface area (m^2/g)	34	37	16	22	20	42	26	162	26	23
Volatile matter	116	77	164	252	75	50	142	67	106	74

387 Data reproduced from UK Biochar Research Centre (http://www.biochar.ac.uk/standard_materials.php; accessed 07/02/2017).

388

389 **Table 2**

390 Fermentation characteristics of different sources of rumen fluid inocula (n=12) used in *in vitro* incubations.

	Mean	SD	Minimum	Maximum
Gas produced (ml/g substrate DM)	267	35.9	212	317
CH_4 produced (ml/g substrate DM)	35	6.1	27	44
Volatile fatty acids produced (mmol/g substrate DM)	1.62	0.373	0.95	2.38
Molar proportions (mmol/mol)				
Acetate	567	25.3	527	599
Propionate	250	36.0	213	312
Butyrate	136	21.2	107	175
Ammonia (mmol/g substrate DM)	1.89	0.437	1.11	2.58

391

392 Table 3

393 Gas and CH_4 production in the absence (control) or presence of biochar prepared from different biomass sources and at different temperatures.

Treatment	Control	Significance‡	Biomass source						Significance‡	
			Miscanthus straw pellets	Oil seed rape straw pellets	Rice husks	Soft wood pellets	Wheat straw pellets	SED	Substrate	Temperature
Gas (ml/g DM)										
550^0	267	P<0.001	252	255	255	256	255	2.6	P=0.11	P=0.058
700^0			254	255	262	258	257			
CH_4 (ml /g DM)										
550^0	35.2	P<0.001	32.1	33.3	33.5	33.4	33.1	0.75	P=0.055	P=0.010
700^0			33.3	33.5	35.0	34.3	33.7			
CH_4 / gas ratio										
550^0	0.159	P=0.021	0.151	0.155	0.152	0.154	0.152	0.0034	P=0.55	P=0.003
700^0			0.155	0.156	0.162	0.158	0.157			

394 ‡Significance for control is for mean effect of biochar calculated by expressing individual values as a proportion of control for each rumen fluid
395 source. For differences between substrate and temperature, there were no interactions between substrate and temperature, nor were there any effects of
396 amount of biochar included in assay.

Table 4

Volatile fatty acid (VFA) production, ammonia (NH3-N) concentrations and pH after incubation in the absence (control) or presence of biochar prepared from different substrates and at different temperatures.

Treatment	Control	Significance‡	Biomass source						Significance‡	
			Miscanthus straw pellets	Oil seed rape straw pellets	Rice husk	Soft wood pellets	Wheat straw pellets	SED	Substrate	Temperature
Total VFA (mmol/g DM)										
550^0	4.86	P=0.15	4.38^a	4.58^a	4.52^{bc}	4.61^{bc}	4.68^{ab}	0.166	P=0.018	P=0.28
700^0			4.51	4.31	4.96	4.83	4.57			
Acetate (mmol /g DM)										
550^0	2.72	P=0.70	2.46	2.58	2.54	2.58	2.64	0.103	P=0.051	P=0.23
700^0			2.54	2.43	2.81	2.73	2.57			
Propionate (mmol /g DM)										
550^0	1.45	P<0.001	1.31^a	1.34^{ab}	1.31^b	1.33^{ab}	1.36^{ab}	0.043	P=0.025	P=0.53
700^0			1.32	1.27	1.42	1.38	1.34			
Butyrate (mmol /g DM)										
550^0	0.58	P=0.021	0.49^a	0.53^{ab}	0.54^c	0.56^c	0.55^{bc}	0.023	P<0.001	P=0.071
700^0			0.54	0.51	0.59	0.59	0.54			
NH3-N (mmol/g DM)										
550^0	1.89	P<0.001	1.03^a	1.32^b	1.58^b	1.51^b	1.45^b	0.072	P<0.001	P=0.007
700^0			1.16	1.55	1.56	1.48	1.58			
pH										
550^0	6.54	P<0.001	6.51	6.52	6.54	6.53	6.54	0.011	P<0.001	P=0.52
700^0			6.51	6.52	6.52	6.54	6.55			

‡Significance for control is for mean effect of biochar calculated by expressing individual values as a proportion of control for each rumen fluid source. For differences between substrates and temperature; there were no interactions between substrate and temperature, nor were there any effects of amount of biochar included in assay. . Means with different superscripts are different (P<0.05).